Translational

Retinal Vascular Rescue of Oxygen-Induced Retinopathy in Mice by Norrin

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Purpose. Wnt-signaling has been implicated in retinal development. The aim of this study was to investigate the possibility of improving retinal vasculature in an animal model of retinopathy by activating Wnt-signaling.

Methods. C57BL/6J mice were evaluated using a model of oxygen-induced retinopathy (OIR). Test animals were divided in three groups and treated at postnatal day (P) 14 with intravitreal injections of Wnt-signaling modulators (respectively, norrin, Dickkopf-related protein 1 [DKK1], and norrin + DKK1) in one eye. A fourth group of animals were treated with injection of PBS in one eye as well and used as a control group. Areas of avascular retina and neovascular tufts in injected (treated) eyes and noninjected fellow eyes were determined in each of the four groups at P17 (3 days after intravitreal injection) and the difference related to these characteristics was obtained among them. To evaluate the effect of norrin on progression of retinopathy, a fifth litter (eight animals) was also treated with norrin and these retinas were evaluated at different time points.

Results. Modulation of Wnt-signaling consistently shows a statistically significant decrease in the avascular area of the retinas. Treatment with norrin (Wnt-signaling activator) or DKK1 (canonical signaling inhibitor) results in a statistically significant reduction of retinal avascular area compared with control eyes. Neovascular tufts were also reduced in treated eyes, albeit to a lesser extent.

Conclusions. Modulation of Wnt-signaling improves retinal vascularization and accelerates vascular recovery after induction of retinopathy in the OIR mouse. Activation of Wnt-signaling (norrin) and inhibition of Wnt-canonical signaling (DKK1) result in similar improvement, indicating that norrin promotes improved vascularization, at least in part, by way of noncanonical Wnt-signaling. (*Invest Ophthalmol Vis Sci.* 2013; 54:222–229) DOI:10.1167/iovs.12-10127

Alterations in norrin function are associated with many pediatric vitreoretinopathies, such as Norrie disease (ND),[1–3] familial exudative vitreoretinopathy (FEVR),[4,5] Coats disease,[6] and retinopathy of prematurity (ROP).[7–9] A unifying characteristic in these diseases is an aberration of retinal vascular development, demonstrating varying degrees of peripheral avascular retina, abnormal vascularization with retinal neovascularization (NV), and subretinal exudation.

At the cellular level, it is widely accepted that disruption of Norrin-Fzd4 signaling is the key causative factor. Frizzled-4 is one of 11 Frizzled transmembrane receptors known to participate in Wnt signaling. Inside the cell, the Wnt signal can activate three pathways: one canonical (Wnt/β-Catenin) and two noncanonical (Wnt/PCP and Wnt/Ca^{++}). There is evidence that norrin may activate all three of these intracellular Wnt pathways.[10–14]

Norrin is a small secreted protein with a cysteine-knot motif.[10,15,16] The cysteine-knot motif is highly conserved in many growth factors including transforming growth factor-β (TGF-β), human chorionic gonadotropin, nerve growth factor, and platelet-derived growth factor.

The structural similarity of norrin to other growth factors suggests that it may have a function in addition to traditional Wnt-signaling, despite the fact that it is best characterized as a Wnt-receptor ligand. This theory is supported by its lack of structural similarity to that of other Wnt proteins.[17]

A previous study demonstrated that endogenous expression of norrin inhibits oxygen-induced retinopathy (OIR) in a mouse model.[18] The purpose of this study was to investigate the possibility of rescuing vascular development with exogenous treatment of norrin in the OIR mouse. We present our findings regarding the therapeutic feasibility of intravitreal injection of the norrin protein and its effect on retinal development.

Materials and Methods

This study adheres to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experiments were performed in an accredited laboratory.

Mouse Model

An OIR model using C57BL/6J mice neonates was used in this study, as previously described.[19] Briefly, at postnatal day (P) 7, mice were exposed to 75% oxygen for 5 consecutive days. At P12, mice were removed from the oxygen chamber and returned to room air. They were divided in three groups and, using a 34-gauge beveled needle (NanoFil; World Precision Instruments, Sarasota, FL), received an intravitreal injection of 1 μL of norrin (Norrie disease protein [NDP], 200 ng; R&D Systems, Minneapolis, MN) (group 1), Dickkopf-related protein 1 (DKK-1, 30 ng; R&D Systems) (group 2), or the combination of both (NDP 200 ng + DKK-1 30 ng) (group 3) in the right eye at P14. Because each litter might have a different level of OIR depending on the weight[20] (despite all animals being euthanized at the same time point), the fellow eye was not injected, being left as an internal control. In a fourth group, mice underwent intravitreal injection of 1 μL of phosphate-buffered saline (PBS) (Sigma, St. Louis, MO) in the right eye as well, to determine the effect of the injection alone, and serve as a

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control for the effect of intravitreal injection. At P17, mice were euthanized and each of the four groups was subdivided into two subgroups according to weight (<5.0 g or >5.1 g).

To determine the effect of norrin on the natural progression and recovery of vessel abnormalities in the OIR model, we tested another group of eight animals. At P14 each animal received an intravitreal injection of norrin (200 ng) in the right eye. The left eye remained as an uninjected control. Two animals were euthanized at P15, P17, P19, and P21.

Histology and Imaging

All eyes were enucleated and fixed in 4% paraformaldehyde (PFA) (Sigma) for 1 hour. Retinas were then isolated, the vasculature was stained with 500 μL lectin solution (10 μg/mL, Isolectin B4-594; Molecular Probes, Eugene, OR) overnight, and whole mounted flat. Images of the whole retinal mounts were taken at ×5 magnification using a fluorescent microscope (Zeiss, Axio Imager; Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The superficial vascular plexus and preretinal neovascular tufts were captured. To evaluate vessel characteristics in higher details, images were also taken in ×10 and ×20 at the transition of avascular and vascularized areas, as well as the mid periphery. To quantify the avascular areas, we used image editor software (CS5; Adobe Photoshop Systems, San Jose, CA) to merge pictures and calculate the areas based on the total number of pixels.[21] Neovascularization was analyzed using image-processing software (SWIFT_NV; Adobe Photoshop Systems) as previously reported.[22] The avascular area and neovascular tufts were established as a percentage of total retinal area.

Statistical Analysis

Based on a previous report,[20] we hypothesized that weight would influence the severity of vascular abnormalities with various interventions at a single time point (P17). To analyze this, we first compared the level of avascular and neovascular areas of all uninjected fellow eyes to the weight of the respective animal. Retinal avascular area and neovascular tufts were compared between fellow eyes of all test groups. Each animal received injection in one eye (norrin, DKK1, combined, or PBS) and the fellow eye served as the control. The results were analyzed by paired *t*-test.

Results

The Role of Weight on Severity of Retinopathy

Although all animals had their retinae evaluated at the same time point (P17), we observed that their weight had a negative correlation with the severity of avascular area (correlation coefficient: –0.48) and a positive correlation with the development of neovascular tufts (correlation coefficient: +0.51) (Figs. 1A, 1B). This suggested that, for the OIR model, the development and progression of the vessel abnormalities are related not only to the age of the animals, but also to their weight.

Avascular Rescue

At P17, the avascular area in untreated eyes average 23.2% of total retinal area in animals weighing <5 g. The animals weighing 5.1 g or more had improved vascularization at the same time point (P17) with an average avascular area of 14.1%, indicating that animals with greater postnatal weight gain have improved vascular recovery (Fig. 1A). Therefore, the analysis of vascular recovery was segregated into pups weighing <5.0 g or ≥5.1 g. Due to the decreased rate of survival of the smaller pups, not all test groups (DKK1 < 5.0 g) were performed. However, the comparable test groups do show similar results.

The analysis of retinal whole mounts at p17, in pups treated with intravitreal injection of norrin, show a statistically significant decrease in the avascular area compared to untreated (uninjected) fellow eyes in pups weighing ≥5.1 g ($P < 0.001$) and <5.0 g ($P = 0.015$), representing a 32% and 27% increase in vascularization, respectively. In contrast, the PBS injection (control group), when compared with the uninjected fellow eye, had minimal impact on vascular growth in both weight groups, demonstrating a 15% (≥5.1 g) and 9.2% (<5.0 g) increase in vascularization, which does not reach statistical significance (Figs. 2, 3A, 3B).

In order to investigate the role of the Wnt/canonical signaling pathway, we inhibited canonical signaling by intravitreal injection of DKK1, which blocks canonical signal transduction by binding the necessary coreceptor, LRP5. Similar to the norrin-treated eyes, inhibition of the Wnt-canonical signaling by DKK1 shows improved vascular recovery, with an average increase of 28% ($P = 0.006$; Fig. 3A). Interestingly, the eyes treated simultaneously with DKK1 and norrin did not result in a statistically significant difference in the avascular area (Figs. 3A, 3B) compared with uninjected eyes in either weight category. The finding that treatment with norrin alone or DKK1 alone demonstrates a statistically significant reduction in the avascular area of the retina suggests that norrin may act on a noncanonical pathway to induce retinal vascular recovery.

Neovascular Changes

The size difference in pups again results in differences in the number of neovascular tufts. Interestingly, the smaller pups have fewer neovascular tufts amounting to approximately one-half as many tufts. This suggests a delay in pathologic changes with failure to thrive (Fig. 1B), similar to that seen in premature infants. Presumably, the smaller pups would show an increased number of neovascular tufts at a later time point as the retina attempts to revascularize and pathologic angiogenesis begins. In general, both weight groups show a trend toward less neovascular changes after treatment with norrin.

In pups with weights < 5.0 g, there is a significant decrease in NV following norrin treatment ($P = 0.02$) compared with control fellow eyes. Mice weighing ≥5.1 g had a slightly different appearance of the NV at P17 in norrin-treated eyes, showing elongated vessels as opposed to the round shape neovessel sprouts seen in untreated eyes, possibly characterizing a step of vessel remodeling before complete revascularization (Fig. 2). Again there is a significant decrease in neovascular tufts in the norrin-treated eyes ($P < 0.001$), as well as eyes treated with DKK1 ($P = 0.006$). Conversely, the combination treatment (DKK1 and norrin) does not result in a significant change in NV in either cohort (Figs. 4A, 4B).

Surprisingly, the PBS-treated eyes in pups weighing ≥5.1 g also show fewer neovascular tufts ($P < 0.001$), although there is not a statistically significant reduction in neovascular tufts in the <5.0 g pups. It is possible that intraocular pressure changes may have some effect on NV, although we would expect to see this effect in the eye receiving combination treatment as well. Alternatively, this finding could be an artifact due to the greater baseline number of tufts in the larger cohorts allowing for more discernible changes.

Progression of Vascular Recovery

Animals treated with norrin (200 ng) and euthanized at different time points (P15, P17, P19, P21) show a significant difference in both avascular area and NV between treated and untreated eyes at each time point except P15 (Fig. 5). In addition, the morphology of the neovascular tufts in P17-

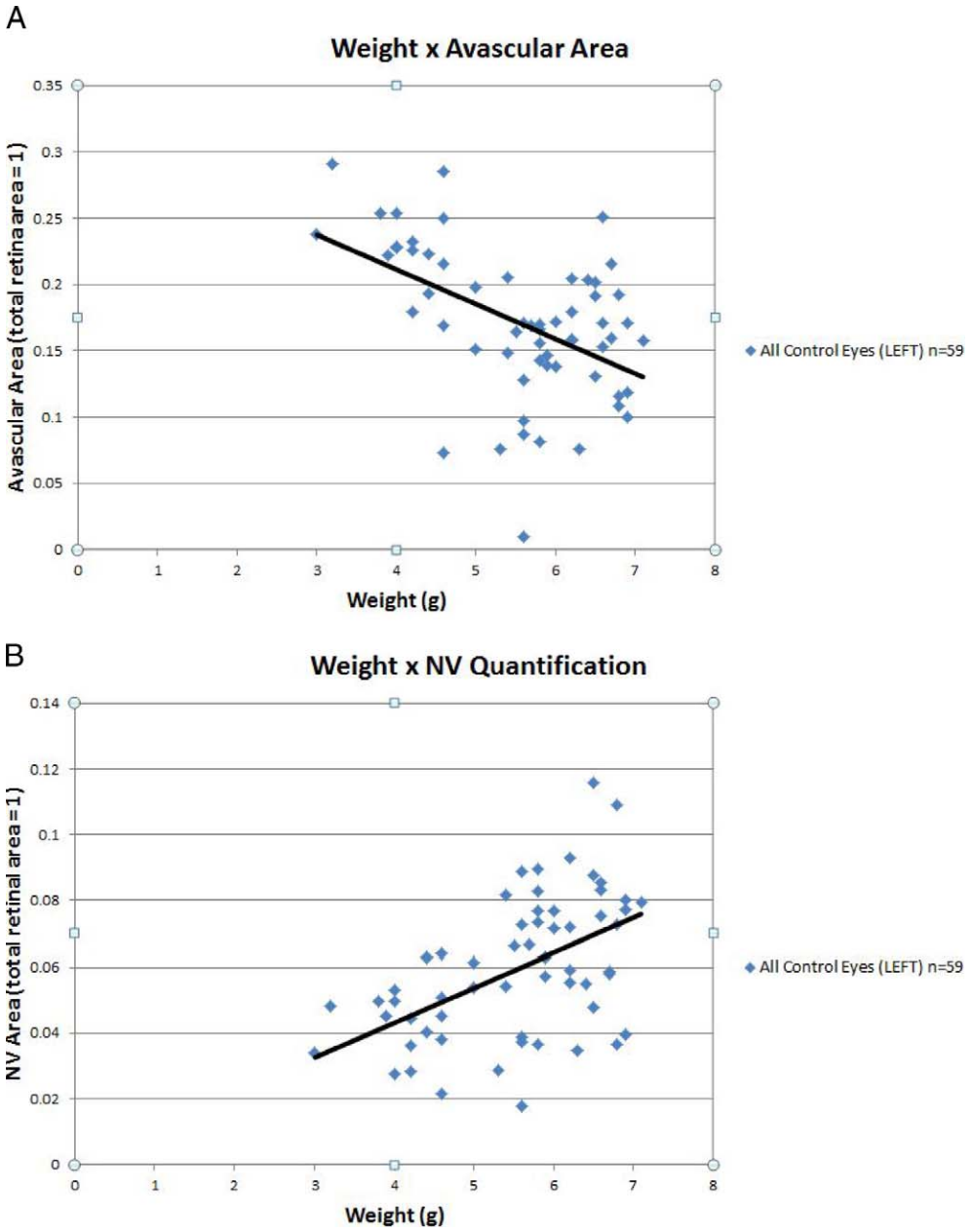

Figure 1. Plot of effect of postnatal weight on (**A**) avascular area of retina and (**B**) neovascular tufts in age-matched mice exposed to 75% oxygen.

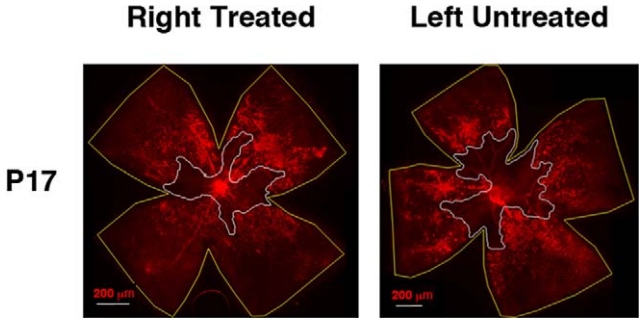

Figure 2. Whole mount of fellow retinas from an OIR mouse at P17. The right eye was treated with norrin; the left eye served as the untreated control.

treated eyes was similar to that of untreated ones at P19, suggesting a faster vascular remodeling with treatment. This trend supports the role of norrin in modulating vascular growth and its positive effect on vascular recovery.

Discussion

In our study, we used an oxygen-induced retinopathy murine model initially described in 1994 by Smith et al.[19] This model shares the two characteristic phases of ROP seen in humans: vasoobliteration (VO) followed by neovascularization (NV).[22] Therefore, OIR in mice represents a reliable animal model for the study of ROP, as well as other retinal vascular diseases.

According to our findings, norrin is able to rescue mouse retinas from retinopathy by promoting stable vascular growth and decreasing the pathologic secondary changes. Importantly, this effect is possible with norrin given as an intravitreal

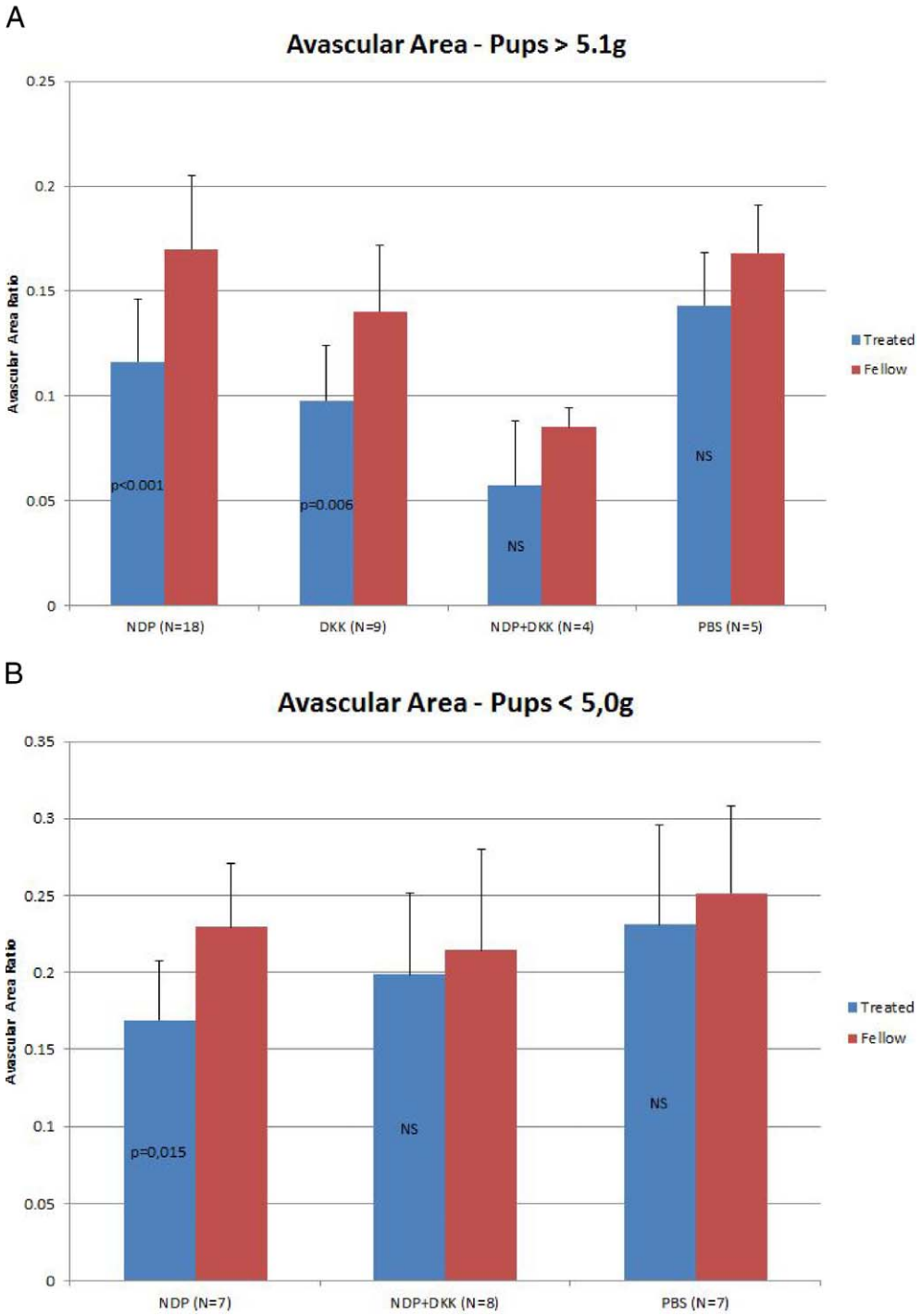

Figure 3. Graph plotting the change in avascular retinas in treated or untreated eyes in mice weighing >5.1 g (**A**) or <5.0 g (**B**).

injection in eyes that have already developed retinopathy. A previous study has shown that endogenous expression of norrin prevents the onset of retinopathy in environments of high oxygen. This same study found that lack of norrin expression may be involved in the development of OIR.[18] Our study is the first to demonstrate the ability of exogenous norrin to reverse a vascular disease process and promote normal retinal vasculature.

Both canonical and noncanonical Wnt signal transduction have been implicated in new blood vessel formation, which requires the coordination of endothelial cell division and the morphogenic movement of vessel expansion.[23,24] In an attempt to understand norrin's mechanism of action we tested DKK1, an inhibitor of the canonical Wnt signaling pathway. We found that injection of DKK1 at p14 significantly enhanced revascularization of the retina by P17. Since DKK1 inhibits LRP5, a coreceptor needed for canonical Wnt signaling, we surmised that noncanonical Wnt signaling was responsible for the DKK1-induced vascular rescue. Several Wnt ligands that are considered to typically activate canonical signaling have been shown to be upregulated in P17 OIR retinas. Chen et al.[25] have reported an increased expression in Wnt ligands, Wnt 3a,

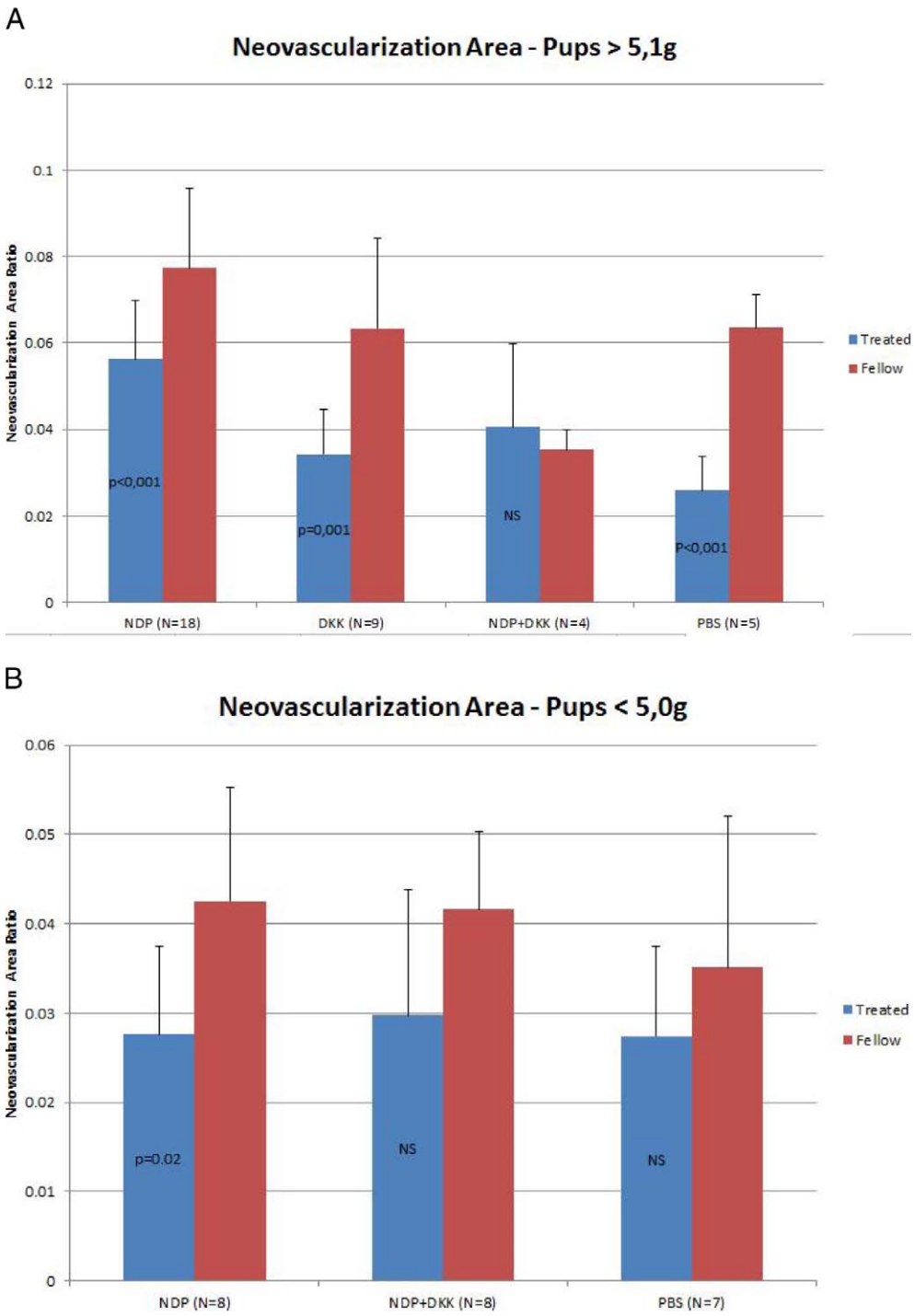

Figure 4. Changes in the number of neovascular tufts in animals with treated and untreated eyes weighing >5.1 g (**A**) or <5.0 g (**B**).

Wnt 7a, and Wnt 10a, in retinas taken from P17 OIR model mice compared with room air controls. Interestingly, they reported no increase in norrin expression in the P17 OIR retinas. The same study also investigated LRP5−/− mice using the OIR and found a decrease in both normal and abnormal vessels at P17. These findings suggest that canonical signal activation plays a role in pathologic angiogenesis.

The improved rescue effect seen with injection of DKK1 led us to speculate that the enhanced vascular recovery seen with injection of norrin alone was also due to inducement of one or both of the noncanonical Wnt pathways. Therefore, we expected to find improved vascular recovery with the coinjection of DKK1 and norrin. To our surprise, this was not the case. Coinjection of DKK1 and norrin counteracted the angiogenic effect seen when either agent was injected alone. We believe that this seemingly contradictory result can be explained by the complexity of Wnt signaling.

Which of the three Wnt intracellular pathways transmits the signal may depend on cellular context. The presence of various receptor complex components may determine which pathway is activated. For example, LRP5 is a requirement for canonical signaling in general and TSPAN12 enhances canonical activa-

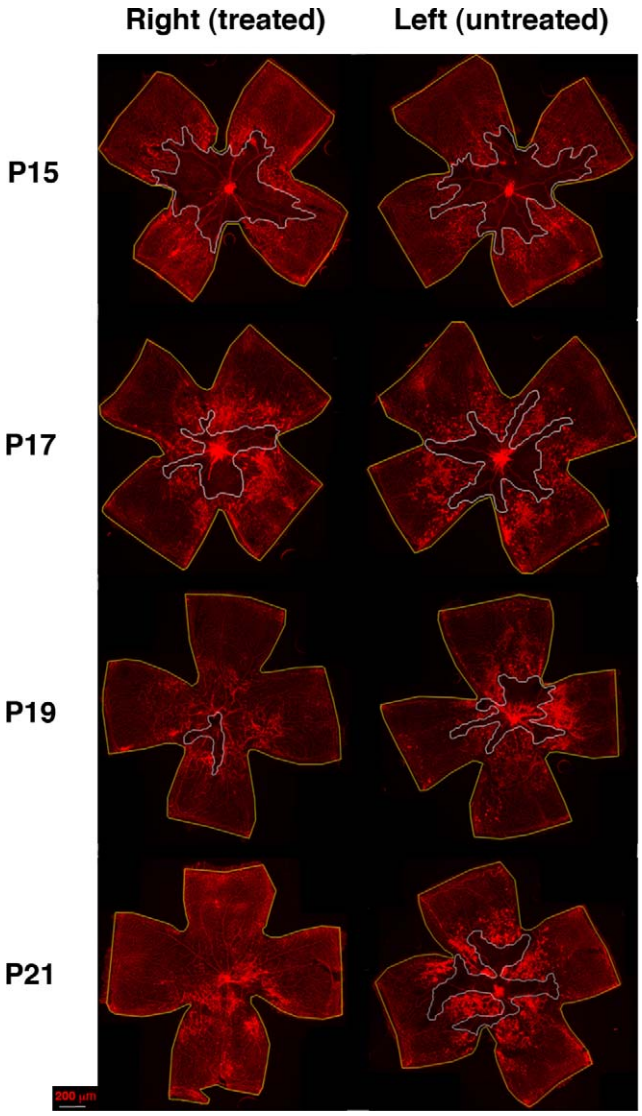

Figure 5. Whole mount of fellow retinas from OIR mice at postnatal days 15, 17, 19, and 21. The right eyes were treated with norrin; the left eye served as the untreated control.

tion by norrin.[14] Perhaps norrin has a greater affinity for the Fzd4 receptor alone, and the effective removal of LRP5 by DKK1 increases norrin's binding to the canonical receptor complex. In the combined (norrin + DKK1) injection, one could envision that norrin binds to a receptor complex that cannot be activated, given that the coreceptor (LRP5) has been bound by DKK1. Therefore, DKK1 binding of LRP5 may result in increased affinity of norrin for the canonical receptor complex, effectively sequestering norrin away from the noncanonical pathways. This would result in decreased noncanonical signaling and defective canonical signaling, essentially canceling any effect of norrin. In this scenario the rescue effect seen with DKK1 alone may be masked by the norrin binding. In other words, the norrin may competitively inhibit binding of the endogenous Wnts to the Fzd4 receptor.

Another complexity that one must consider when evaluating the Wnt intracellular pathways is the regulation of one pathway by others. For instance, it is generally accepted that the noncanonical pathways antagonize the Wnt/β-Catenin pathway[24] and inhibiting canonical signaling can activate Wnt/PCP signaling.[26] For example, Wnt5a activation of the Wnt/JNK (PCP) pathway has been shown to inhibit Wnt/β-Catenin signaling in in vitro reporter assays.[27]

Finally, norrin may also act as a growth factor during retinal development given its structural similarity to that of several growth factors. This is supported by the fact that it is an antagonist to TGF-β.[28] Additionally, mutations affecting the receptors for TGF-β (TGFRB1 and TGFRB2) result in a retinal phenotype of FEVR, further supporting the possibility that norrin also functions as a growth factor.

Both norrin and DKK1 reduce the number of neovascular tufts in the OIR model, paralleling the results seen in the promotion of vascular recovery. Again, the combined treatment with norrin and DKK1 results in cancellation of any treatment effect. Vascular endothelial growth factor (VEGF) has been shown to be pathologically elevated in infants with ROP.[29] Retinal endothelia cell (REC) culture studies have shown that norrin is able to inhibit the tight junction loosening effect of VEGF on RECs (unpublished data). Perhaps this mode of action is involved in the norrin reduction of NV.

A decrease in neovascular tufts was also seen in the PBS-treated eyes of the larger pups. Indeed, all injected eyes show a trend toward fewer neovascular tufts compared with uninjected eyes. Intravitreal injection per se might have some effect on the vitreous–retina interface, but does not induce significant changes in neovessel formation. In our study, if there was a significant injection effect, this would be noticed in the group we injected with a combination of norrin and DKK1, which did not occur. For this reason, we believe that the significant result seen in PBS cohort (>5.1 g) was due to the relatively lower number of mice ($n = 5$) rather than to a true protective effect. The low-birth-weight pups also show fewer neovascular tufts in the PBS-treated eyes but it does not reach statistical significance. One reason for the difference between the PBS groups may be the relatively few neovascular tufts developing in the low-birth-weight pups, resulting in a trend toward fewer tufts with injection of any substance, but the low starting number of tufts may prohibit detection of small changes. Historically, evaluation of neovascular tufts results in less consistent data and has value in assessing trends and supporting data but cannot stand alone.

In the OIR murine model used in this study, the maximum severity of the proliferative phase is reached at P17, marked by the greatest extent of pathologic NV and associated plasma leakage from the preretinal neovessels.[20] Our data suggest that animal weight plays a role in the severity and timing of onset of OIR as well. Comparing the uninjected fellow eye of all animals with the weight of the corresponding animal, we observed that mice with lower weight presented fewer neovascular tufts and larger avascular areas at P17, indicating that low weight promotes a delay in both vascular growth and pathologic angiogenesis. Other investigators have also reported that animals with lower weight at birth or slower weight gain velocity, show a delayed onset of OIR and a prolonged course of vascular abnormalities. Stahl et al.[20] showed that mice with body weights between 5 and 7.5 g at P17 displayed the highest amount of NV, whereas mice with either <5 g or >7.5 g body weight at P17 showed significantly lower severity of NV. With these findings, it seems reasonable that mice with lower weight at birth and slower growth behave like normal-weight pups but at an earlier postnatal age, resulting in the postponement of vascular abnormalities involved in OIR, such as neovascular tufts.

Once we identified the vascular differences seen with weights we sought to understand the natural progression of vascular growth and pathologic changes in the presence and absence of norrin treatment. Therefore, littermates were treated with intravitreal norrin in the right eye at P14 (fellow eye uninjected) and evaluated at sequential time points (P15,

P17, P19, P21). Despite the low sample number, we had interesting findings: at P15, as we would expect, the avascular area was large and the neovascular tufts were very few, both in treated and untreated eyes, representing vascular obliteration (Fig. 5). By P17 the avascular area was smaller in treated eyes and the neovascular tufts were fewer as compared with the fellow (uninjected) eye. The norrin-treated eye at P19 shows almost full revascularization of the retina. This is in stark contrast to the uninjected fellow eye. The most remarkable difference was observed at P21. In addition to significant vascular recovery in the norrin-treated eyes, compared with uninjected control eyes, the P21 pups had a significant difference in weight gain (6.7 and 4.7 g). The normal-weight mouse (6.7 g) had fewer vascular abnormalities in both eyes (norrin and uninjected), as expected, given the natural vascular recovery in the OIR model at this time point. However, the lower-weight mouse (4.7 g) showed a large difference in vascular recovery between the fellow eyes. The norrin-treated eye had nearly complete revascularization, whereas the untreated fellow eye still possessed a very large avascular area and neovascular tufts. These findings again support that poor weight gain postpones the development and natural course of OIR. However, norrin has an even more remarkable effect in these low-weight animals, possibly by promoting and accelerating normal vessel development. This finding has great importance in regard to premature infants at the highest risk for the development of retinopathy of prematurity and reinforces the potential use of norrin in humans.

The highly localized expression of norrin in the retina, cochlea, and central nervous system during development suggests a highly specific role for norrin in the appropriate maturation of these particular tissues. There are other nonspecific Wnt ligands that are able to bind both the Fzd4 and LRP5 (receptor and coreceptor for norrin), but these have been shown to be upregulated during pathologic angiogenesis.[25] Clinical studies and animal models clearly show that lack of norrin expression in the eye results in severe abrogation of retinal development, indicating that Wnt pathway activation alone (by other Wnt ligands) is not sufficient for normal retinal development and vasculaturization.[30–33]

Based on these findings, norrin may represent a unique molecule that is able to function as both a Wnt-ligand and a growth factor and regulate angiogenesis in a fashion that mimics that seen in the developing eye. This has significant implication in the treatment of many eye diseases characterized by anomalous vasculature, including avascular retina, vascular permeability, capillary drop-out, and NV. These conditions are seen in the inherited vitreoretinopathies, such as FEVR, Norrie disease, and persistent fetal vasculature, as well as retinopathy of prematurity, diabetic retinopathy, and retinal artery and vein occlusions. Future studies will aim to evaluate these diseases as potential therapeutic targets for norrin.

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References

1. Schuback DE, Chen ZY, Craig IW, Breakefield XO, Sims KB. Mutations in the Norrie disease gene. *Hum Mutat*. 1995;5: 285–292.
2. Meindl A, Berger W, Meitinger T, et al. Norrie disease is caused by mutations in an extracellular protein resembling C-terminal globular domain of mucins. *Nat Genet*. 1992;2:139–143.
3. Berger W, van de Pol D, Warburg M, et al. Mutations in the candidate gene for Norrie disease. *Hum Mol Genet*. 1992;1: 461–465.
4. Shastry BS, Hejtmancik JF, Plager DA, Hartzer MK, Trese MT. Linkage and candidate gene analysis of X-linked familial exudative vitreoretinopathy. *Genomics*. 1995;27:341–344.
5. Chen ZY, Battinelli EM, Fielder A, et al. A mutation in the Norrie disease gene (NDP) associated with X-linked familial exudative vitreoretinopathy. *Nat Genet*. 1993;5:180–183.
6. Black GC, Perveen R, Bonshek R, et al. Coats' disease of the retina (unilateral retinal telangiectasis) caused by somatic mutation in the NDP gene: a role for norrin in retinal angiogenesis. *Hum Mol Genet*. 1999;8:2031–2035.
7. Talks SJ, Ebenezer N, Hykin P, et al. De novo mutations in the 5′ regulatory region of the Norrie disease gene in retinopathy of prematurity. *J Med Genet*. 2001;38:E46.
8. Hutcheson KA, Paluru PC, Bernstein SL, et al. Norrie disease gene sequence variants in an ethnically diverse population with retinopathy of prematurity. *Mol Vis*. 2005;11:501–508.
9. Hiraoka M, Berinstein DM, Trese MT, Shastry BS. Insertion and deletion mutations in the dinucleotide repeat region of the Norrie disease gene in patients with advanced retinopathy of prematurity. *J Hum Genet*. 2001;46:178–181.
10. Xu Q, Wang Y, Dabdoub A, et al. Vascular development in the retina and inner ear: control by Norrin and Frizzled-4, a high-affinity ligand-receptor pair. *Cell*. 2004;116:883–895.
11. Descamps B, Sewduth R, Ferreira Tojais N, et al. Frizzled 4 regulates arterial network organization through noncanonical Wnt/planar cell polarity signaling. *Circ Res*. 2012;110:47–58.
12. Yao R, Natsume Y, Noda T. MAGI-3 is involved in the regulation of the JNK signaling pathway as a scaffold protein for frizzled and Ltap. *Oncogene*. 2004;23:6023–6030.
13. Robitaille J, MacDonald ML, Kaykas A, et al. Mutant frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. *Nat Genet*. 2002;32:326–330.
14. Junge H, Yang S, Burton J, et al. TSPAN12 regulates retinal vascular development by promoting norrin, but not Wnt-induced FZD4/β-catenin signaling. *Cell*. 2009;139:299–311.
15. Berger W. Molecular dissection of Norrie disease. *Acta Anat (Basel)*. 1998;162:95–100.
16. Black G, Redmond RM. The molecular biology of Norrie's disease. *Eye*. 1994;8:491–496.
17. McDonald NQ, Hendrickson WA. A structural superfamily of growth factors containing a cystine knot motif. *Cell*. 1993;73: 421–424.
18. Ohlmann A, Seitz R, Braunger B, Seitz D, Bosl MR, Tamm ER. Norrin promotes vascular regrowth after oxygen-induced retinal vessel loss and suppresses retinopathy in mice. *J Neurosci*. 2010;30:183–193.
19. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci*. 1994;35: 101–111.
20. Stahl A, Chen J, Sapieha P, et al. Postnatal weight gain modifies severity and functional outcome of oxygen-induced proliferative retinopathy. *Am J Pathol*. 2010;177:2715–2723.
21. Connor KM, Krah NM, Dennison RJ, et al. Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. *Nat Protoc*. 2009;4:1565–1573.
22. Stahl A, Connor KM, Sapieha P, et al. Computer-aided quantification of retinal neovascularization. *Angiogenesis*. 2009;12:297–301.
23. Zeng G. Orientation of endothelial cell division is regulated by VEGF signaling during blood vessel formation. *Blood*. 2007; 109:1345–1352.
24. Dejana E. The role of Wnt signaling in physiological and pathological angiogenesis. *Circ Res*. 2010;107:943–952.

25. Chen J, Stahl A, Krah NM, et al. Wnt signaling mediates pathological vascular growth in proliferative retinopathy. *Circulation*. 2011;124:1871–1881.
26. Rao TP, Kühl M. An updated overview on Wnt signaling pathways. A prelude for more. *Circ Res*. 2010;106:1798–1806.
27. Mikels A, Nusse R. Purified Wnt5a protein activates or inhibits β-Catenin-TCF signaling depending on receptor context. *PLoS Biol*. 2006;4:570–582.
28. Tamm E, Ohlmann A, inventors; Universitat Regensburg, assignee. Norrin in the treatment of diseases associated with an increased TGF-beta activity. European patent EP20090798920. September 28, 2011.
29. Sonmez K, Drenser K, Capone A, Trese M. Vitreous levels of stromal cell-derived factor 1 and vascular endothelial growth factor in patients with retinopathy of prematurity. *Ophthalmology*. 2008;115:1065–1070.
30. Robitaille J, Zheng B, Wallace K, et al. The role of Frizzled-4 mutations in familial exudative vitreoretinopathy and Coats disease. *Br J Ophthalmol*. 2011;95:574–579.
31. Chen Y, Hu Y, Zhou T, et al. Activation of the Wnt pathway plays a pathogenic role in diabetic retinopathy in humans and animal models. *Am J Pathol*. 2009;175:2676–2685.
32. Wu WC, Drenser K, Trese M, Capone A Jr, Dailey W. Retinal phenotype-genotype correlation of pediatric patients expressing mutations in the Norrie disease gene. *Arch Ophthalmol*. 2007;125:225–230.
33. Chen J, Stahl A, Krah NM, et al. Retinal expression of Wnt-pathway mediated genes in low-density lipoprotein receptor-related protein 5 (Lrp5) knockout mice. *PLoS One*. 2012;7:e0030203.